Review

Respirable antisense oligonucleotides: a new drug class for respiratory disease

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Received: 4 October 2000
Revisions requested: 16 October 2000
Revisions received: 10 November 2000
Accepted: 10 November 2000
Published: 18 December 2000

Respir Res 2001, **2**:5–9

(Print ISSN 1465-9921; Online ISSN 1465-993X)

Abstract

Respirable antisense oligonucleotides (RASONs), which attenuate specific disease-associated mRNAs, represent a new class of respiratory therapeutics with considerable potential. RASONs overcome previous obstacles that have impeded the development of antisense therapeutics targeting diseases in other organ systems. RASONs are delivered directly to the target tissue via inhalation; their uptake seems to be enhanced by cationic properties inherent in pulmonary surfactant, and, because of the markedly different target properties of mRNA and proteins, they can have very long durations of effect compared with traditional drugs targeting the protein of the same gene. RASONs contain chemical modifications that decrease their degradation by cellular nucleases. However, total insensitivity to nucleases is probably not an optimal design criterion for RASONs, because moderate nuclease sensitivity can prevent their systemic delivery, decreasing the potential for systemic toxicity. EPI-2010 is a 21-mer phosphorothioate RASON that attenuates bronchoconstriction, inflammation and surfactant depletion in preclinical models of human asthma, has a duration of effect of seven days, and seems to undergo minimal systemic delivery.

Keywords: asthma, DNA medicines, respiratory disease

Introduction

Antisense oligonucleotides have been called 'the next great wave of the biotechnology revolution' and the 'pharmacology of the future'. Certainly from a theoretical point of view, the antisense approach, in which the target pharmacophore is a specific sequence found in a specific mRNA, should have several advantages over traditional pharmaceuticals, which target proteins. The most obvious potential advantage is that interdiction occurs at a point more proximal to the cause of the disease – the (usually) discordantly expressed mRNA – rather than the protein already participating in the disease process. Other potential advantages of targeting mRNA instead of protein include the following:

1. Increased specificity and avidity. Antisense oligonucleotides can bind ('hybridize') to their mRNA targets with much greater specificity and with much greater avidity than can traditional drugs to their protein targets. This is because the sum of the hydrogen bonding between the oligonucleotide and its mRNA target exceeds by several orders of magnitude the Van der Waals and other forces with which traditional drugs bind to proteins.

2. Ability to prescreen for non-target hybridization. Because an antisense oligonucleotide targets a specific sequence (essentially a one-dimensional target) rather than a complex three-dimensional amino acid

CNS = central nervous system; RASON = respirable antisense oligonucleotide.

domain, it becomes possible to screen in advance of toxicology studies for hybridization potential to non-target mRNAs. This can be accomplished for known genes by database scanning, and for unknown genes by northern and Southern blotting techniques. Such an advantage in predictive toxicology is not available in the development of traditional drugs, which target complex three-dimensional domains within proteins. The complexity of such protein domains at present prevents any meaningful a priori identification of interaction of a drug candidate with non-target proteins.

3. Markedly different target properties. The attenuation of a target mRNA population might require much longer periods to recover to disease-causing levels than inhibition at the protein level, as exemplified by studies on EPI-2010 (see below). This feature can translate into markedly longer durations of effect after the attenuation of a specific mRNA via the antisense approach in comparison with the inhibition, with traditional drugs, of the protein coded for by the same mRNA.

4. Rapid development. Antisense therapeutics can be designed and tested much more quickly and rationally than traditional drugs, with antisense libraries consistently showing success rates several orders of magnitude greater than for traditional libraries. The success rate for traditional libraries, that is, the chance of finding, via large-scale screening, a compound that will bind to a target protein, has been cited as being as high as 0.02% [1]. Compare this with a consistently reported success rate of approximately 10% for antisense libraries. The potential benefits, in terms of a reduction in development times and an acceleration of the rate of making drugs available to the patients who need them, are enticing from both economic and medical perspectives.

Thus, there are many reasons why antisense oligonucleotides could be a potential breakthrough in technology for the more rapid and more economic development of highly specific, effective drugs. Nevertheless, it should also be pointed out that not every potential respiratory target is likely to represent a target for which the antisense approach is optimal. Considerations such as quantity and half-life of the mRNA and protein of the target are likely to be important determinants of whether the antisense approach will work or not. Other considerations related to the successful therapeutic application of this class of molecules include the potential to induce immunologic effects with particular nucleotide backbone structures or sequence motifs.

The practical application of antisense oligonucleotide technology has been impeded by several factors. The first of these, the ubiquitous presence of nucleases that can degrade oligonucleotides quite readily, has been successfully addressed by a number of chemical modifications that inhibit susceptibility to cellular nucleases [2,3]. Recent advances in oligonucleotide chemistry have included chimeric oligonucleotides that incorporate multiple chemical strategies to enhance hybridization to target mRNA and/or to reduce toxicity. A second and perhaps more significant problem, that has impeded the practical application of the theoretical potential of the antisense approach, is the difficulty in delivering oligonucleotide drugs to the target tissue in amounts large enough to be efficacious but small enough to be without significant toxicity. Local delivery represents one way to solve this problem, and its effectiveness is exemplified by Fomivirsen™, the first commercially available antisense oligonucleotide [4]. As we show below, respirable antisense oligonucleotides (RASONs) represent a particularly attractive form of local delivery, creating an exciting opportunity for the development of an array of new pharmaceuticals targeting respiratory diseases [5].

RASONs

One problem that has hindered the development of antisense therapeutics is that parenteral administration of these drugs has required high doses for acceptable levels of efficacy in the target tissue because the target tissue is generally distant from the point of administration. Non-antisense side effects related to backbone modifications (eg hypotension with phosphorothioate backbones), to specific sequence motifs (eg gene activation by quartets of guanine base occurring within the oligonucleotide), or to immune stimulation (unmodified cytosines occurring 5′ to guanines stimulate B cell mediated immunosurveillance) occur in a dose-dependent fashion, a factor that suggests strongly that minimum efficacious doses should be used.

The lung offers an exceptional target for antisense oligonucleotide delivery. RASONs can be delivered directly to the target tissue, by inhalation, thereby achieving a bolus dose directly to the target site. The lung is also uniquely lined with surfactant, a material that seems to substantially enhance the uptake and distribution of oligonucleotides throughout the pulmonary tissues. How might surfactant contribute to oligonucleotide uptake and distribution? Surfactant is primarily composed of zwitterionic lipids that at lung pH have cationic properties. Cationic lipids are very well known to enhance the uptake of oligonucleotides into cells [6]. In fact, it is often difficult to obtain cellular permeation of oligonucleotides under conditions *in vitro* without the addition of cationic lipids [7]. When oligonucleotides are inhaled, they seem to be adsorbed by surfactant, resulting in a reformulation of the oligonucleotide in such a way as to make it amenable to facile uptake within the cells and tissues of the respiratory tract. Autoradiograms of inhaled RASONs indicate that penetration occurs even to the deeper tissues of the lung, and surgical dissection of

bronchial smooth muscle to primary, secondary and tertiary bronchi in RASON-treated animals, followed by quantification of target receptors, confirms this [8•]. The end result of such surfactant-facilitated uptake of oligonucleotides is a rather marked reduction in what constitutes an effective dose. RASONs have been found to be effective at doses several orders of magnitude less than is usual for other forms of parenteral administration. The mechanism of this interaction between surfactant and an inhaled oligonucleotide have not been elucidated in detail, but probably involve processes more complex than the simple adsorption on cationic lipids observed under conditions *in vitro*.

One problem that has plagued traditional pulmonary drugs is that it has been difficult to limit their effects to the lung. Systemic toxicity is the dose-limiting consideration for a large number of respiratory drugs currently on the market, and was the cause of discontinued development for many others. RASONs offer a potential solution to this problem as well, because their nuclease sensitivity can be titrated by altering their chemistry to make them sufficiently nuclease resistant to be effective, but not so nuclease resistant that they could escape the lung undegraded. With the use of this strategy, RASONs are available for hybridization to their target, but then are degraded before they can enter the wider circulation. Alternatively, RASONs can be designed to augment their nuclease resistance substantially, permitting systemic delivery to occur after their inhalation. Although the design of such 'super-resistant' oligonucleotides would permit the targeting of receptors found in the periphery, this strategy eliminates the advantage of reduced systemic toxicity that less nuclease-resistant inhaled RASONs offer.

RASONs thus seem to offer an effective solution to the problem of how to deliver just enough oligonucleotide to the target tissue to attenuate the target mRNA effectively without producing systemic toxicity.

EPI-2010

EPI-2010 is a 21-mer phosphorothioate RASON targeting the adenosine A_1 receptor. It functions by degrading the mRNA of the adenosine A_1 receptor, preventing the synthesis of new receptor protein. There is therefore a lag between the administration of EPI-2010 (estimated to be less than 12 h) and sufficient loss of preformed receptor protein to obtain a biological response.

Adenosine is thought to be an important mediator of asthma, and the adenosine A_1 receptor is an important target for asthma therapeutic intervention, for several reasons:

1. Adenosine levels are above normal in the bronchial lavage fluid of asthmatic patients [9] and in animal models of asthma [10].
2. Adenosine upregulation has been shown to be sufficient to induce a powerful inflammatory response in mice [11••].
3. Asthmatic patients and animals show hypersensitivity to inhaled adenosine [12••].
4. Adenosine A_1 receptor levels are upregulated both in asthmatic patients [13•] and in animal models of asthma [14].
5. Adenosine A_1 receptor levels are upregulated in bronchial smooth muscle cells as one of the earliest responses to antigen stimulation [15•].
6. Adenosine A_1 receptor is involved in all three of the major sequelae of asthma: bronchoconstriction, inflammation, and surfactant depletion [16].

Traditional drugs targeting the adenosine A_1 receptor have not been successfully developed, despite large commitments of time and expense, primarily owing to systemic toxicity of these drugs. The RASON approach might make possible the successful attenuation of such difficult targets because of the ability to design into oligonucleotide structures sufficient nuclease sensitivity to ensure almost total pulmonary degradation. Such pulmonary degradation should prevent any non-pulmonary antisense-mediated attenuation of the target receptor, although it might not inhibit any toxicities associated with the chemical nature of the oligonucleotide (eg the phosphorothioate or other backbone chemistry employed).

EPI-2010 targets the initation codon of the adenosine A_1 receptor mRNA (–9 to +11), and is one of the most effective antisense oligonucleotides ever reported in a setting *in vivo* [8•]. We have shown that aerosolized EPI-2010 potently and selectively attenuates adenosine A_1 receptor expression *in vivo*. It is effective at single delivered inhaled doses on the order of 50 μg/kg. The half life of EPI-2010 in the lung is less than 24 hours, but its duration of effect (its ability to block hyper-responsiveness to inhaled adenosine) is 6.8 days (range 4–11 days). This disparity suggests that aspects of the target such as quantity and half-life of the target mRNA, and/or the quantity and half-life of the protein for which the mRNA codes, have a major role in determining the effectiveness of the RASON approach toward a specific target. EPI-2010 is now undergoing phase I clinical trials.

Primate models of human asthma

One aspect of the RASON approach that is potentially problematic is that oligonucleotide-based medicines are species-specific, in that variations in the DNA sequence of genes increase as phylogenetic distance increases. Thus, animal models are generally not applicable to pharmaco-

logical testing of RASONs designed for use in humans. Even single-base non-homologies are sufficient to reduce the hybridization potential markedly. Differences between the target human sequence and the corresponding sequence in an animal model can be accommodated only by using antisense oligonucleotides having a sequence structure completely homologous to that of the animal. Only in rare circumstances is the human sequence identical to that for an animal used in a model system, unless the animal is a primate. Only primates have consistently high levels of homology to human DNA; for example, cynomolgus monkey DNA is at least 97.8% homologous to that of humans. Primate models of human disease are therefore much preferred for the pharmacological testing of new antisense constructs, not only because non-human primate disease might mimic human disease more closely, but also because antisense constructs designed against human genes will show complete homology to the non-human primate gene with much greater consistency than for any other species. EpiGenesis has created the TruPrimate™ model of human asthma to address this need.

RASON-based target validation

The combination of the RASON approach and the TruPrimate™ model can also be used to validate or invalidate putative respiratory targets. For example, techniques such as differential display can be used to identify genes whose pulmonary expression seems to be anomalous in the disease state in comparison with the healthy state, and RASONs can be used to attenuate the expression of the anomalously expressed gene. Pulmonary function test results before and after RASON intervention can then be assessed to determine whether or not the anomalously expressed gene is an appropriate subject for the initiation of a drug discovery program.

One important criterion for accurate target validation, not only in the respiratory tract but also in the central nervous system (CNS) and elsewhere, is that the antisense oligonucleotides used in the process should cleanly ablate the function of the targeted gene product without inducing obfuscating side effects. When oligonucleotides are degraded, they release nucleotides and nucleosides of all four major bases (adenine, guanine, cytosine, and thymidine/uridine). The release of adenosine as an oligonucleotide degradation product is of extreme concern because of adenosine's major role in a host of physiological processes ranging from vasoconstriction to control of sinoatrial rhythm [16,17].

The release of bioactive adenosine from oligonucleotide degradation can have very important consequences in hyper-responsive airways and also in applications involving the CNS (either direct microinjection into specific brain regions, or intrathecal administration). For example, individuals with asthma show a nearly universal hypersensitivity to inhaled adenosine and adenosine monophosphate, and adenosine released as a product of antisense oligonucleotide breakdown is capable of activating this response. (An exception is EPI-2010, in which the receptor mediating adenosine sensitivity is downregulated by RASON administration and is therefore not available for adenosine stimulation). The administration of a RASON containing adenosine might thus cause dose-limiting bronchoconstriction during the process of oligonucleotide degradation.

Similarly, the release of adenosine into the CNS during antisense oligonucleotide degradation can cause the stimulation of any or all of the four adenosine receptors (A_1, A_{2A}, A_{2B}, or A_3), potentially causing effects in any of the more than 40 different physiological processes in which these receptors have a role in the CNS [16,17]. Such effects, occurring in parallel to the functional gene ablation caused by the antisense oligonucleotide, could obscure the effects of the antisense oligonucleotide, preventing meaningful target validation data from being obtained.

To avoid this problem, EpiGenesis has developed *des*Adenosine™ EpiGene Screen™ antisense oligonucleotides, which do not release adenosine when they are degraded and therefore cannot modulate the activity of adenosine receptors. This technology is being applied to validate or invalidate putative drug discovery candidate targets in both the respiratory tract and the CNS.

Conclusions

Inhaled RASONs might permit the therapeutic potential of the antisense approach to be realized in a consistent way. The unique presence of surfactant in the lung probably underlies this consistency, because surfactant, in the manner of cationic lipids, seems to facilitate the pulmonary uptake and distribution of inhaled oligonucleotides. Two potential advantages of the RASON approach over traditional respiratory medicines include an attenuation of the disease process at a point more proximal to its cause, the anomalously expressed gene, and a reduction in systemic side effects owing to the ability to engineer RASONs such that they are not likely to leave the lung in an undegraded form. Although not every gene relevant to respiratory disease might have characteristics amenable to attenuation by the antisense approach, RASONs have the potential to markedly improve therapeutic outcomes in a wide array of such diseases.

Acknowledgements

Parts of this work were funded by NIH SBIR grants 1 R43 HL7716-01A1 and 2 R44 HL57716-02. In the interest of full disclosure, JWN wishes it to be known that he is an employee of EpiGenesis Pharmaceuticals, and that he therefore possesses a vested interest in proprietary products and technologies of the company, including EPI-2010, the TruPrimate™ model of human asthma, and *des*Adenosine™ EpiGene Screen™ technology.

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